PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 04 December 2000 (04.12.00)	
International application No. PCT/JP00/03022	**Applicant's or agent's file reference** YCT-497
International filing date (day/month/year) 11 May 2000 (11.05.00)	**Priority date** (day/month/year) 11 May 1999 (11.05.99)
Applicant YOSHIZAKO, Kimihiro et al	

1. The designated Office is hereby notified of its election made:

[X] in the demand filed with the International Preliminary Examining Authority on:

05 October 2000 (05.10.00)

[ ] in a notice effecting later election filed with the International Bureau on:

2. The election [X] was

[ ] was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Kiwa Mpay
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

is covalently attached to a base matrix. A temperature responsive polymer is indirectly affinity bound to the ligand groups by multi-point attachment, i.e. the attachment of the thermo-responsive polymer is depending on the prior attachment of the ligands to the base matrix. By changing the temperature the ligand becomes more or less prone to affinity bind to its target substance.

Hofman et al., (WO 8706152) describe a separation method in which the ligand is attached to a temperature responsive polymer. Binding and elution of the target substance occur at the same side of the critical solution temperature. For the term critical solution temperature se further under the discussion about thermo-responsive polymers.

There are also a number of publications describing chromatography based on separation material comprising stimulus-responsive polymers but without having a ligand covalently attached to the temperature-responsive polymer. Gewehr et al (Macromolecular Chemistry and Physics 193 (1992) 249-256) describe gel chromatography on porous silica beads coated with a temperature-responsive polymer. Hosoya et al (Anal. Chem. 67 (1995) 1907-1911); Yamamoto et al. (Proc. 114th National Meeting of the Pharmaceutical Society of Japan, Tokyo (1994) 160; Kanazawa et al (Yakugaku Zasshi 117 (10-11) (1997) 817-824; Kanazawa et al (Anal. Chem. 68(1) (1996) 100-105); Kanazawa et al (Anal. Chem. 69(5) (1997) 823-830); Kanazawa et al (J. Pharm. Biomed. Anal. 15 (1997) 1545-1550); Yakushiji et al (Langmuir 14(16) 1998) 4657-466268); Kanazawa et al (Trends Anal. Biochem. 17(7) (1998) 435-440); Yakushiji et al (Anal. Chem. 71(6) 1999) 1125-1130); Grace & Co (EP 534016); Okano (JP 6-108643) describe

PATENT COOPERATION TREATY

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

TOMITA, Hiroyuki
YUASA AND HARA
Section 206, New Ohtemachi Bldg,
2-1 Ohtemachi 2-chome
Chiyoda-ku
Tokyo 100-0004
JAPON

PCT

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	09.08.2001
Applicant's or agent's file reference: YCT-497	**IMPORTANT NOTIFICATION**

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/JP00/03022	11/05/2000	11/05/1999

Applicant
JAPAN CHEMICAL INNOVATION INSTITUTE et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Gregoire, J-P Tel.+49 89 2399-8041

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
YCT-497		

International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/JP00/03022	11/05/2000	11/05/1999

International Patent Classification (IPC) or national classification and IPC
B01J20/32

Applicant
JAPAN CHEMICAL INNOVATION INSTITUTE et al.

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 5 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 2 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☐ Priority
   - III ☐ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
05/10/2000	09.08.2001

Name and mailing address of the international preliminary examining authority:

European Patent Office
D-80298 Munich
Tel. +49 89 2399 - 0 Tx: 523656 epmu d
Fax: +49 89 2399 - 4465

Authorized officer

Nazario, L

Telephone No. +49 89 2399 8137

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/JP00/03022

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-3,5-26	as originally filed	
4,4a	with telefax of	15/06/2001

Claims, No.:

1-9	as originally filed

Drawings, sheets:

1/1	as originally filed

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

   These elements were available or furnished to this Authority in the following language: , which is:

   - ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
   - ☐ the language of publication of the international application (under Rule 48.3(b)).
   - ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

   - ☐ contained in the international application in written form.
   - ☐ filed together with the international application in computer readable form.
   - ☐ furnished subsequently to this Authority in written form.
   - ☐ furnished subsequently to this Authority in computer readable form.
   - ☐ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
   - ☐ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	7
	No:	Claims	1-6, 8-9
Inventive step (IS)	Yes:	Claims	
	No:	Claims	1-9
Industrial applicability (IA)	Yes:	Claims	1-9
	No:	Claims	

2. Citations and explanations
   see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
see separate sheet

Re Item V

Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Reference is made to the following documents:

   D1: JP-A-09 049830 (Patent Abstracts of Japan)
   D2: JP-A-08 103653 (Patent Abstracts of Japan)
   D3: JP-A-07 136505 (WPI abstract)
   D4: JP-A-07 135957 (WPI abstract)
   D5: EP-A-0 534 016

2. D1-D4 disclose separating materials comprising a stimulus-responsive polymer and a substance having specific affinity on the surface of a support matrix (see abstracts). D1 clearly discloses that the polymer (or magnitude of structural change) is selected according to the target to be separated and D2 also discloses a number of physical changes that are suitable, e.g. heat. The use of these materials in chromatography is also disclosed (e.g. D4). The cited documents anticipate the subject-matter of claim 1. In its present form, independent claim 1 relates to a material wherein a stimulus-responsive polymer and an affinity substance are attached to a support. Such a characterisation also includes the materials disclosed in D1-D4.

   Therefore, the subject-matter of claims 1-6, 8 and 9 is not novel and does not fulfill the requirements of article 33(2) PCT.

3. The subject-matter of claim 7 is not disclosed in D1-D4 and is therefore novel (article 33(2) PCT). However, such a distinguishing feature is banal and would be an obvious selection for the skilled man in the art. For example, D5 discloses such support material in combination with a stimulus-responsive polymer (D5, abstract, col. 1, lines 3-1, col. 4, lines 23-33, col. 5, lines 20-29, col. 7, line 50 to col. 8, line 22).

   Therefore, the subject-matter of claim 7 does not involve an inventive and does not fulfill the requirements of article 33(3) PCT.

4. It is well known in the art to use affinity compounds to separate target substances (see, for example, D1-D5). The applicant's attention is drawn to the fact that all the essential features (i.e. elements of the solution of the technical problem) have to be included in the independent claim and a particular effect (e.g. improved recovery ratio) has to be directly related to a distinguishing feature (or features).

Re Item VIII

Certain observations on the international application

1. To fulfill the requirements of article 6 PCT the following have to be addressed:

1.1. In claim 1, a bracketed term is included such a formulation renders the claim unclear.

1.2. Although claims 2-4 are dependent on claim 1, which relates to a material, these claims do not contain any additional technical features of the material but effectively relate to method features of how to modify the affinity and/or environment of the material.

1.3. Claim 8 is dependent on claim 1, however it effectively relates the use of the material. Therefore, it would seem appropriate to redraft the claim so that the subject-matter can be clearly perceived.

PATENT COOPERATION TREATY

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INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
YCT-497		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/JP 00/ 03022	11/05/2000	11/05/1999

Applicant

JAPAN CHEMICAL INNOVATION INSTITUTE et al.

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [ ] furnished subsequently to this Authority in written form.

   [ ] furnished subsequently to this Authority in computer readble form.

   [ ] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [ ] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PC[redacted] 00/03022

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 B01J20/32 B01D15/08

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 B01J B01D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
PAJ, WPI Data, EPO-Internal, CHEM ABS Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATENT ABSTRACTS OF JAPAN vol. 1997, no. 06, 30 June 1997 (1997-06-30) & JP 09 049830 A (TERUMO CORP), 18 February 1997 (1997-02-18) abstract ---	1-5,8,9
X	PATENT ABSTRACTS OF JAPAN vol. 1996, no. 08, 30 August 1996 (1996-08-30) & JP 08 103653 A (TERUMO CORP), 23 April 1996 (1996-04-23) abstract --- -/--	1-5,8,9

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
31 August 2000	06/09/2000

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hilgenga, K

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Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International Application No
PC[redacted] 00/03022

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
X	DATABASE WPI Section Ch, Week 199530 Derwent Publications Ltd., London, GB; Class A97, AN 1995-227588 XP002145661 & JP 07 136505 A (TERUMO CORP), 30 May 1995 (1995-05-30) abstract	1-5,8,9
X	DATABASE WPI Section Ch, Week 199530 Derwent Publications Ltd., London, GB; Class A96, AN 1995-227387 XP002145662 & JP 07 135957 A (TERUMO CORP), 30 May 1995 (1995-05-30) abstract	1-5,8,9
A	WO 94 15951 A (MATTIASSON) 21 July 1994 (1994-07-21) cited in the application	
A	EP 0 534 016 A (W.R. GRACE & CO.) 31 March 1993 (1993-03-31)	
A	GALAEV I Y ET AL: "Temperature-induced displacement of proteins from dye-affinity columns using an immobilized polymeric displacer" JOURNAL OF CHROMATOGRAPHY A,NL,ELSEVIER SCIENCE, vol. 684, no. 1, 28 October 1994 (1994-10-28), pages 37-43, XP004174364 ISSN: 0021-9673 cited in the application	

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INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/EP 00/03022

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
JP 09049830 A	18-02-1997	NONE	
JP 08103653 A	23-04-1996	NONE	
JP 7136505 A	30-05-1995	NONE	
JP 7135957 A	30-05-1995	NONE	
WO 9415951 A	21-07-1994	AU 5894294 A	15-08-1994
EP 534016 A	31-03-1993	JP 4006463 A	10-01-1992

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 7 : B01J 20/32, B01D 15/08 — A1

(11) International Publication Number: **WO 00/67901**

(43) International Publication Date: 16 November 2000 (16.11.00)

(21) International Application Number: PCT/JP00/03022

(22) International Filing Date: 11 May 2000 (11.05.00)

(30) Priority Data:
11/130267 11 May 1999 (11.05.99) JP

(71) Applicants *(for all designated States except US)*: JAPAN CHEMICAL INNOVATION INSTITUTE [JP/JP]; 22–13, Yanagibashi 2–chome, Taito–ku, Tokyo 111–0052 (JP). JAPAN as represented by DIRECTOR GENERAL OF AGENCY OF INDUSTRIAL SCIENCE AND TECHNOLOGY [JP/JP]; 3–1, Kasumigaseki 1–chome, Chiyoda–ku, Tokyo 100–0013 (JP).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: YOSHIZAKO, Kimihiro [JP/JP]; 4–24–202, Higashi 2–chome, Tsukuba=shi, Ibaraki 305–0046 (JP). AKIYAMA, Yoshikatsu [JP/JP]; 15–18, Togoshi 1–chome, Shinagawa–ku, Tokyo 142–0041 (JP). OKANO, Teruo [JP/JP]; 12–12, Kounodai 6–chome, Ichikawa–shi, Chiba 272–0827 (JP). UENO, Katsuhiko [JP/JP]; 670–50, Hirooka, Tsukuba–shi, Ibaraki 305–0042 (JP).

(74) Agent: TOMITA, Hiroyuki; Yuasa And Hara, Section 206, New Ohtemachi Bldg., 2–1, Ohtemachi 2–chome, Chiyoda–ku, Tokyo 100–0004 (JP).

(81) Designated States: AU, CA, JP, US, European patent (DE, FR, GB, IT, SE).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: AFFINITY–CONTROLLING MATERIAL WITH THE USE OF STIMULUS–RESPONSIVE POLYMER AND SEPARATION/PURIFICATION METHOD WITH THE USE OF THE MATERIAL

(57) Abstract

An affinity–controlling material, wherein a stimulus–responsive polymer and an affinitive substance (ligand) having affinity for a target substance are independently attached, preferably covalently, to a support matrix is provided. The material is capable of separating and purifying a target substance such as a physiologically active substance under a physical stimulus while keeping at least one condition other than temperature (for example, pH value of solution, organic solvent concentration or salt concentration) constant by using a support matrix capable of preventing nonspecific adsorption of proteins and achieving an excellent separation performance.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

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AFFINITY-CONTROLLING MATERIAL WITH THE USE OF STIMULUS-RESPONSIVE POLYMER AND SEPARATION/PURIFICAITON METHOD WITH THE USE OF THE MATERIAL

Technical Field

This invention relates to an affinity-controlling material comprising (a) a stimulus-responsive polymer and (b) an affinitive substance (ligand) having affinity for a target substance that is present in a solution in contact with the material. By subjecting the material to a stimulus, for instance a physical stimulus, the chemical and/or physical environment provided around the ligand can be changed thereby changing the affinity between the ligand and the target substance. The change in environment is typically related to a conformational change in the stimulus-responsive polymer.

The term "solution" as used herein means liquids, typically containing dissolved buffer components and salts (ions). Typical solutions have as the liquid component: a solvent such as water, an organic solvent and mixtures thereof. Organic solvents include in particular those that are miscible with water, for example, water-miscible alcohols, acetonitrile, tetrahydrofuran, etc and mixtures thereof. The terms "change in affinity", "changing the affinity" etc refer to the apparent affinity, i.e. the affinity that can be measured under the conditions applied.

Affinity-controlling material will further on also be called separation medium or separation material.

The present invention further relates to an affinity-

controlling material with which a desired target substance (metal ions, drugs, biological components, etc.) can be removed or separated and/or purified from mixtures containing other substances.

The present invention furthermore relates to a method for separating and purifying target substances (metal ions, drugs, biological components, etc.) with the use of the above-mentioned affinity-controlling material. The method preferably comprises keeping at least one condition other than temperature (for example, pH value of solution, organic solvent concentration or salt concentration) constant.

Background Art

There have previously been employed ion exchange chromatography, reversed phase chromatography, and other affinity chromatography principles and various batch-wise protocols based on affinity binding as means for efficaciously separating and purifying biological components, drugs, etc from mixtures of substances. With the recent advances in biotechnology, a number of novel physiologically and biologically active substances including recombinant proteins have been developed. At the same time there has been an increased need for improved methods for separating and purifying these substances without unacceptable losses in biological and physiological activity.

Separation and/or purification of a target substance from a mixture of substances by affinity chromatography and other adsorption based separation techniques typically encompass a

binding step (adsorption step) and a release step (desorption or eluting step). Compared to the binding step, the release step typically requires a change in the composition of the liquid in contact with the separation medium. Illustrative examples for accomplishing the appropriate change are adding an organic solvent to a mobile phase, elevating the salt concentration of the mobile phase, or changing the pH value of the mobile phase. This also applies to the turbulent or non-turbulent liquid phases used in batch-wise procedures. These operations result in an increased risk for inactivation of physiologically active substances. Even if an active substance often may be separated and purified by conventional chromatographic techniques without unacceptable losses in activity, the organic solvent, salt, etc added to the mobile/liquid phase should in most cases at least be partially removed from a purified or isolated target substance. This leads to an additional risk for lowering the activity and/or recovery/yield of the target substance.

During the last decade there has been an interest in combining so called stimulus-responsive polymers with chromatographic techniques and other techniques based on binding or partition of a desired substance to an insoluble separation medium of the type used in chromatography.

Recently, separation media comprising ion exchanging groups that are covalently attached to stimulus-responsive polymers have been described. See for instance JP application 140722/98 with corresponding patent application WO 99/61904

Galaev et al (J. Chromatog. A 684 (1994) 37-43 and WO 94/154951 describe temperature elution of a target substance in a chromatographic system in which a plurality of ligand groups

is covalently attached to a base matrix. A temperature responsive polymer is indirectly affinity bound to the ligand groups by multi-point attachment, i.e. the attachment of the thermo-responsive polymer is depending on the prior attachment of the ligands to the base matrix. By changing the temperature the ligand becomes more or less prone to affinity bind to its target substance.

Hofman et al., (WO 8706152) describe a separation method in which the ligand is attached to a temperature responsive polymer. Binding and elution of the target substance occur at the same side of the critical solution temperature. For the term critical solution temperature se further under the discussion about thermo-responsive polymers.

There are also a number of publications describing chromatography based on separation material comprising stimulus-responsive polymers but without having a ligand covalently attached to the temperature-responsive polymer. Gewehr et al (Macromolecular Chemistry and Physics 193 (1992) 249-256) describe gel chromatography on porous silica beads coated with a temperature-responsive polymer. Hosoya et al (Anal. Chem. 67 (1995) 1907-1911); Yamamoto et al. (Proc. 114th National Meeting of the Pharmaceutical Society of Japan, Tokyo (1994) 160; Kanazawa et al (Yakugaku Zasshi 117 (10-11) (1997) 817-824; Kanazawa et al (Anal. Chem. 68(1) (1996) 100-105); Kanazawa et al (Anal. Chem. 69(5) (1997) 823-830); Kanazawa et al (J. Pharm. Biomed. Anal. 15 (1997) 1545-1550); Yakushiji et al (Langmuir 14(16) 1998) 4657-466268); Kanazawa et al (Trends Anal. Biochem. 17(7) (1998) 435-440); Yakushiji et al (Anal. Chem. 71(6) 1999) 1125-1130); Grace & Co (EP 534016); Okano (JP 6-108643) describe

reversed phase chromatography on matrices covered by a thermoresponsive polymer for the separation of biomolecules. The matrices may be porous. The hydrophobic groups utilized are inherent in the polymer as such. There is no ligand that has been covalently attached to the polymer after polymerisation.

Certain aspects of the general ideas of performing separations on chromatography on (a) a separation medium covalently functionalized with a conjugate between a stimulus-responsive polymer and an affinity ligand and (b) a separation medium functionalized by separate/independent attachment of a stimulus-responsive polymer and an affinity ligand were presented at National meetings of Chemical Society of Japan on March 28, 1999 and on May 27 1999 (SPSJ, the Society of Polymer Science, Japan, Annual Meeting, Abstract p583) respectively.

Separation media are often in the form of particles that may be porous and non-porous. The particles typically comprise a base matrix to which the ligand is attached directly or indirectly, for instance via a spacer. The particle material may be a synthetic polymer such as crosslinked polymerisates of styrenes, acrylates/methacrylates and the like. However, polystyrene particles and polymethacrylate particles per se are relatively hydrophobic and therefore often exhibit a pronounced non-specific adsorption of various substances that may be present together with a desired target substance. When such particles are to be used as a support in affinity-based separations, it is therefore necessary to make them sufficiently hydrophilic in order to minimize the hydrophobic/non-specific adsorption. To improve the separation and purification

performance of particles, it is often favorable to use particles of uniform size (monosized or monodispersed particles). Although there has been a demand for hydrophilic synthetic polymeric supports in form of porous particles of uniform size for a long time, there have been known few methods for producing the same in practice.

Disclosure of Invention

The objects of the invention are to provide solutions to the problems discussed above thereby enabling improved separation methods and separation materials.

The present inventors have conducted intensive studies and found that an affinity-controlling material/separation medium can be synthesized by attaching a stimulus-responsive polymer and an affinitive substance (ligand) of a target substance independently to a support, i.e. via separate linking structures. The present inventors have further found that a target substance adsorbed by the above-mentioned affinity-controlling material can be desorbed under a physical stimulus, such as a temperature change, while keeping at least one conditions other than the stimulus concerned constant. If, for instance, the stimulus is a temperature change, the condition(s) to be kept constant may be selected amongst, for example, pH, organic solvent concentration, salt concentration etc. The present inventors have furthermore found that the bonding ability between a ligand and a target substance depends on the length of a spacer by which the ligand is attached to a support/base matrix or on the size of the stimulus-responsive polymer bonded to the support.

The present inventors have also conceived to use hydrophilic porous polymer particles that may be of uniform size (monosized = monodispersed) as support material in the invention. In this part of the invention the inventors thus have conceived to use particles obtained by polymerisation of monomer emulsions/suspensions obtained by the membrane emulsification method. This embodiment also includes to chemically treat the particles with acidic or basic substances in order to introduce hydrophilic groups and/or groups that will enable covalent attachment of the ligand and/or the stimulus-responsive polymer, for instance hydroxy and/or amino groups. The chemical treatment requires that the starting material (monomer) used in the membrane emulsification method exhibits a reactive group that is able to react with the acidic or basic substance utilized. Typical reactive groups have been epoxy groups. The present invention has been completed based on these findings.

Accordingly, the present invention relates to an affinity-controlling material/separation medium of the type defined in the introductory part. One of the major characteristic features is that the stimulus-responsive polymer and the ligand are attached by separate/independent links to a base matrix. When changing the level and/or intensity of the appropriate stimulus for the polymer from one side of the critical level/intensity to the other side, there is caused a reversible change in the affinity between the ligand and its target substance. The stimulus may be a physical stimulus and is in the experimental part typified by a temperature change.

The present invention further relates to an affinity-controlling material wherein the affinity of an affinative

substance of a target substance is reversibly changed by changing the chemical or physical environment of a stimulus-responsive polymer under a physical stimulus while keeping at least one condition other than the changed condition constant, i.e. subjecting the thermo-responsive polymer to a change in one physical stimulus while keeping at least one of the other stimulus constant. If the changed stimulus is the temperature, then at least one condition/stimulus except the temperature is maintained essentially constant (for example, pH, organic solvent concentration or salt concentration).

The present invention furthermore relates to an affinity-controlling material/separation medium as defined above wherein the affinity between the ligand and the target substance depends on

(a) the length of the spacer attaching the ligand to the base matrix or

(b) the size, for instance as reflected in molecular weight, of the stimulus-responsive polymer.

The present invention further relates to an affinity-controlling material/a separation medium as defined above wherein the support (base matrix) comprises hydrophilic porous polymer particles preferably having a uniform particle size and/or being produced by polymerisation of a monomer emulsion/suspension obtained from the membrane emulsification method. Hydrophobic particles exhibiting functional groups that are reactive with acidic or basic substances, may be rendered less hydrophobic by reaction with this kind of substances. Typical examples of reactive groups are epoxy groups, i.e. if the membrane emulsification method involves polymerisation the

starting monomer contains an epoxy group.

The present invention further relates to utilization of the above-mentioned affinity-controlling materials as a chromatographic packing.

An additional embodiment of the invention is a method for the separation of one or more target substances from a liquid sample (solution) (liquid I). This embodiment comprises the steps of

(a) bringing a liquid sample (liquid (I)) containing a target substance in contact with a separation medium (including a chromatographic packing) which is functionalized with a ligand which is capable of affinity binding to the target substance, said contact being under conditions permitting binding of said target substance to said ligand;

(b) contacting said carrier with a liquid (II) not containing said target substance under conditions such that the target substance is released from said ligand to liquid (II).

Between steps (a) and (b) the liquid sample is preferably separated from the separation medium. After step (b), liquid (II) may be separated from the separation medium. The target substance may, if so desired, be worked up from liquid II. The separation medium may be washed between step (a) and step (b).

The liquids typically have been aqueous for target substances that are biologically and/or physiologically active molecules, e.g. bioorganic molecules having structures selected amongst nucleotide structure (including nucleic acids), polypeptide structure (including proteins), carbohydrate structure, steroid structure etc.

This embodiment of the invention is characterized in that

(i) said separation medium comprises a support/base matrix to which a stimulus-responsive polymer as defined elsewhere in this specification and the ligand are linked separately, and

(ii) subjecting in step (a) and at least during binding of the target substance to the ligand, the separation media to a stimulus at a level/intensity at which the stimulus-responsive polymer is in a conformation enhancing binding of the target substance to the ligand, and

(iii) subjecting in step (b) and at least during release of the target substance from the ligand, the separatory material to a stimulus at a level/intensity at which the stimulus-responsive polymer is in a conformation hindering binding of the target substance to the ligand.

Preferably the same kind of stimulus is referred to steps (a) and (b). Compared to step (a), the level/intensity of the stimulus in step (b) is on the opposite side of the critical level/intensity for the stimulus-sensitive polymer used. The process can be made cyclic in case step a is repeated after step b, typically after separate washing/regeneration steps and equilibration steps.

Various embodiments of the inventive method may be carried out in a batch-wise or a chromatographic mode. Chromatographic modes, for instance, may be carried out by permitting the various liquids in plug flow (mobile phase) to pass through a bed of the separation medium while subjecting the bed to the appropriate stimulus for the individual steps and the stimulus-responsive polymer that is attached to the base matrix. The bed may be a

porous monolith or a bed of packed or fluidised particles. Batch-wise modes concern suspended particles in combination with turbulent flow and/or turbulent liquids.

Brief Description of Drawings

Fig. 1 provides a chromatogram showing control of the affinity of BSA by BC-10 with temperature change.

Best Mode for Carrying Out the Invention

Stimulus-responsive polymer

The physical stimulus to be used in the present invention is exemplified by temperature.

Depending on the particular stimulus-responsive polymer used other stimulus may apply, for instance, light, magnetic field, electrical field, pH etc. Stimulus-responsive polymers are often called "intelligent polymers".

Stimulus-responsive polymers are characterized in that they upon being subjected to the correct kind and intensity or level of a stimulus undergo a conformational and reversible change of their physico-chemical properties. The change may be a switch from a pronounced hydrophobicity to a pronounced hydrophilicity or vice versa. The exact level/intensity the required stimulus at which the switch occurs is called critical level or critical intensity of the stimulus and will depend on the structure of the polymer and often also on other conditions (solvent, solutes such as salts etc). The most wellknown and most utilized polymers of this kind respond to heat (thermo-responsive or temperature-responsive polymers). Temperature-responsive polymers are recognized by a sharp temperature limit

at which they switch from a pronounced hydrophilic state to a pronounced hydrophobic state and vice versa. For temperature-responsive polymer in solution the change in conformation/physico-chemical properties occurs at the so-called critical solution temperature (CST).

For a temperature responsive polymer in aqueous media there is a lower critical solution temperature (LCST) or an upper critical solution temperature (UCST). For a polymer having a LCST, the polymer changes from a hydrophilic conformation to a hydrophobic conformation when the temperature is passing the LCST from below. For a polymer having an UCST, the change is the opposite when the temperature is passing the UCST from below. The exact value of the LCST and UCST depend on the polymer and also on other conditions applied (solvent, other solutes etc).

As discussed above one of the characteristic features of the invention when a temperature-sensitive polymer is used is that the binding to and the release from the ligand are performed at opposite sides of an applicable CST.

The stimulus-responsive polymer to be used in the invention preferably has an insignificant affinity for the target substance compared to the affinity between the target substance and the ligand attached to the support. Preferably there is no significant affinity between the ligand and the stimulus-responsive polymer.

Examples of the stimulus-responsive polymer to be used in the present invention include poly(N-substituted acrylamide) such as poly(N-isopropyl acrylamide), poly(N-substituted methacrylamide) such as poly(N-isopropyl methacrylamide), poly(N,N-disubstituted acrylamide), poly(N,N-disubstituted

methacrylamide), polymethyl vinyl ether, poly(ethylene oxide-propylene oxide) copolymer, polyvinyl alcohol derivatives typified by partly saponified polyvinyl alcohol and cellulose derivatives typified by methyl cellulose. It is also possible to introduce reacting functional groups (for example, amino, carboxyl or hydroxyl groups) into the stimulus-responsive polymer so as to covalently attach the stimulus-responsive polymer to the support/base matrix.

Ligands

Ligands may be attached to the base matrix via affinity bonds or via covalent bonds, preferably the latter. According to the present inventive concept it is not via the stimulus-responsive polymer.

One typical kind of ligands affinity binds to the target substance by more or less pure ionic (electrostatic) interactions. Alternatively the binding includes more complex interactions such as in conventional affinity binding (affinity adsorption). For ionic interactions, the ligands comprise positively or negatively charged entities (ion exchange; the immobilised entity being selected among primary, secondary, tertiary and quaternary ammonium, sulphonate, sulphate, phosphonate, phosphate, carboxy etc groups). More complex interactions are illustrated by the ligand being an individual affinity member in the pairs,

(a) antibodies and antigens/haptens,

(b) lectins and carbohydrate structures,

(c) IgG binding proteins and IgG,

(d) polymeric chelators and chelates,

(e) complementary nucleic acids.

Affinity members also include entities participating in catalytic reactions, for instance enzymes, enzyme substrates, cofactors, cosubstrates etc. Members of cell-cell and cell-surface interactions and a synthetic mimetics of bioproduced affinity members are also included. The term ligand also includes more or less complex organic molecules that binds through affinity to complex biomolecules, for instance having oligo or polypeptide structure (including proteins), oligo and polynucleotide structure (including nucleic acids), oligo- or polysaccharide structures etc.

Further examples of ligands to be used in the present invention include dyes such as CIBACRONE BLUE F3G-A™ (manufactured by Fluka) and other complex dyes, iminodiacetic acid, sugar chains such as glucose, proteins such as heparin and lectin, biotin, benzamidine, lysine, arginine, peptides and DNA. It is also according to the invention possible to control the bonding ability of the target substance by covalently bonding the affinitive substance of the target substance to the support via a spacer such as a bivalent alkyl group or an ethylene oxide group.

The base matrix (e.g. chromatographic packings)

The separation medium to be used in the inventive method comprises a base matrix (carrier) which may be based on organic and/or inorganic material. In case the liquid used is aqueous, the base matrix is preferably hydrophilic. This in particular applies to target substances that are biomolecules of the kind discussed above.

The base matrix is preferably based on a polymer, which preferably is insoluble and more or less swellable in water, preferably to a gel. Hydrophobic polymers that have been derivatized to become hydrophilic are included in this definition. Suitable polymers are polyhydroxy polymers, e.g. based on polysaccharides, such as agarose, dextran, cellulose, starch, pullulan, etc. and completely synthetic polymers, such as polyacrylic acid amide, polymethacrylic acid amide, poly(hydroxyalkylvinyl ethers), poly(hydroxyalkylacrylates) and polymethacrylates (e.g. polyglycidylmethacrylate), polyvinylalcohols and polymers based on styrenes and divinylbenzenes, and copolymers in which two or more of the monomers corresponding to the above-mentioned polymers are included. Polymers, which are soluble in water, may be derivatized to become insoluble, e.g. by cross-linking and by coupling to an insoluble body via adsorption or covalent binding. Hydrophilic groups can be introduced on hydrophobic polymers (e.g. on copolymers of monovinyl and divinylbenzenes) by polymerization of monomers exhibiting groups which can be converted to OH, or by hydrophilization of the final polymer, e.g. by adsorption of suitable compounds, such as hydrophilic polymers.

Suitable inorganic materials to be used in base matrices are silica, zirconium oxide, graphite, tantalum oxide etc.

Preferred base matrices lack groups that are unstable against hyrolysis, such as silan, ester, amide groups and groups present in silica as such. Preferred base matrices contain functional groups that can be used for attaching covalently the stimulus-responsive polymer and/or the ligand. This kind of

functional groups are illustrated by hydroxy, carboxy, amino groups etc.

The matrix may be porous or non-porous. This means that the matrix may be fully or partially permeable (porous) or completely impermeable to the compound to be removed (non-porous).

The pores may have sizes ≥ 0.1 μm, such as ≥ 0.5 μm, by which is meant that a sphere ≥ 0.1 μm respective ≥ 0.5 μm in diameter is able to pass through. An applied liquid may be able to flow through this kind of pore system (convective pore system). In case the support matrix is in form of beads packed to a bed, the ratio between the pore sizes of the convective pore system and the diameter of the particles typically is in the interval 0.01-0.3, with preference for 0.05-0.2. Pores having sizes ≥ 0.1 μm, such as ≥ 0.5 μm, are often called macropores.

The base matrix may also have pores with sizes ≤ 0.5 μm, such as ≤ 0.1 μm by which is meant that only spheres with diameters ≤ 0.5 μm, such as ≤ 0.1 μm, can pass through. Pores having sizes ≤ 0.5 μm, such as ≤ 0.1 μm, are often called micropores.

In one embodiment of the invention, the base matrix is in the form of irregular or spherical particles with sizes in the range of 1-1000 μm, preferably 5-50 μm for high performance applications and 50-300 μm for preparative purposes. Particles to be used may be monodisperse (monosized) or polydispersed (polysized). By the term monodispersed particles is meant a particle populations having more than 95% of the particles with sizes within their mean diameter ± 5%, which in the context of the present invention contemplate the expression particles of

uniform size. Polydispersed particles encompass other populations of particles.

The base matrix may also be in form of a monolith having at least macropores as defined above. Alternative geometric forms are the interior walls of tubes and the like

The stimulus responsive polymer and the ligand as defined above may be attached to the outer surfaces and/or on the interior surfaces (macropore and/or micropore surfaces) of the base matrix. As discussed above the stimulus responsive polymer and the ligand may be attached to the base matrix by physical adsorption and/or covalent attachment, preferably the latter.

It is particularly preferable to use as the support/base matrix hydrophilic porous polymer particles having a uniform particle size, which are produced by the membrane emulsification method followed by a chemical treatment with an acidic substance or a basic substance as discussed above.

The membrane emulsification method as used in the present invention is a method which comprises passing a first liquid through a glass membrane, preferably made of glass, into a second liquid which is mot miscible with the first liquid, thus forming droplets of an essentially size in the second liquid. This method is described in, for example, S. Omi, K. Katami, A. Yamamoto and M. Iso. J. Appl. Polym. Sci., 51 (1994) 1-11. In case the first liquid contains a polymerizable monomer and the droplets are subjected to polymerization, particles will form in the second liquid.

Thus the preferred the support materials (base matrices) according to the inventor's novel finding is produced in the following manner: A liquid mixture (first liquid) is prepared

from a monomer, which serves as the starting material for polymer particles, and a diluent, etc. Next, one side of a porous glass membrane is filled with this liquid mixture and the opposite side with a second liquid which is not miscible with the first liquid. Pressure is the applied to the liquid mixture so that it passes through the membrane and forms droplets in the second liquid. For instance the first liquid may be immiscible with water and the second liquid aqueous containing an emulsion stabilizer, etc. to give an emulsion consisting of droplets of an essentially uniform size. Subsequently, polymerization is carried out by, for example, heating to thereby give latex particles having a uniform particle size. Provided that the monomer contains a group reactive with an acidic or basic substance, the latex particles can be stirred in a solution containing this kind of substances to give hydrophilic porous polymer particles. During this post-treatment, reactive functional groups such as amino groups can be introduced into the support. By using these reactive functional groups, the stimulus-responsive polymer or the ligand can be covalently attached to the support.

Examples of the monomer to be used in producing the hydrophilic porous polymer particles having a uniform particle size include glycidyl acrylate, glycidyl methacrylate, diacrylates (for example, ethylene diacrylate), dimethacrylates (for example, ethylene dimethacrylate), glycidyl vinylbenzyl ether and divinylbenzene. It is also possible to combine these monomers.

The diluent to be used in the production of the hydrophilic porous polymer particles having a uniform particle size may be an arbitrary compound as long as it is not polymerizable with

the monomers used. Examples thereof include aromatic solvents/compounds, such as toluene and aliphatic compounds such as dodecane.

Examples of the acidic substance or basic substance to be used in the production of the hydrophilic porous polymer particles having a uniform particle size include sulfuric acid, hydrochloric acid, nitric acid, acetic acid, sodium hydroxide, ammonia and aliphatic diamines such as 1,6-diaminohexane.

Manufacture of the separation material of the present invention

The affinity-controlling material/separation material according to the present invention can be produced by, for example,

(a) a method which comprises covalently attaching a stimulus-responsive polymer or a copolymer thereof to a support and then covalently attaching an affinitive substance of a target substance to the support; or

(b) a method which comprises covalently attaching the affinitive substance of the target substance to the support and then covalently attaching the stimulus-responsive polymer or a copolymer thereof to the support; or

(c) a method which comprises covalently attaching the affinitive substance of the target substance and the stimulus-responsive polymer or a copolymer thereof respectively to the support at the same time.

Examples

The present invention is illustrated below in more detail with reference to the following examples, but is not to be

construed as being limited thereto.

Example 1

1. Synthesis of stimulation-responsive polymer

N-isopropylacrylamide (20 g), 3-mercaptopropionic acid (0.18 g), and 2,2'-azobis(4-cyanovaleric acid) (0.27g) were dissolved in tetrahydrofuran (200 ml). The resulting solution was placed in a polymerization tube. Oxygen was removed from the solution by the freezing and thawing deaeration method. The polymerization was performed at 60°C for 2 hours. Poly(N-isopropylacrylamide) having a carboxyl group at one end of its molecule was reprecipitated using diethyl ether as solvent.

The molecular weight of the obtained polymer was determined by gel permeation chromatography (GPC) and end-group analysis. GPC was performed using dimethylformamide containing 10 mM lithium bromide as a mobile phase, a column á-3000 (TOSOH Co., Japan) column, and polystyrene as a standard reference material. The number average molecular weight and the weight average molecular weight of the synthesized poly(N-isopropylacrylamide) were found to be about 4,500 and 10,000, respectively. The carboxyl terminal groups of the synthesized poly(N-isopropylacrylamide) were determined by end group analysis with a 0.01N sodium hydroxide solution. As a result, the number average molecular weight was about 5000. It was thus confirmed that the number average molecular weight of the synthesized poly(N-isopropylacrylamide) determined by GPC is essentially the same as that determined by end group analysis.

The synthesized poly(N-isopropylacrylamide)(10 g), N-hydroxysuccinimide (0.25 g), and N,N'-dicyclohexyl-

carbodiimide (0.45 g) were dissolved in tetrahydrofuran (60 ml), and the resulting solution was stirred at room temperature for 12 hours. The resulting precipitate was collected by filtration and reprecipitated in diethyl ether to give poly(N-isopropylacrylamide) whose carboxyl group at one end is esterified with N-hydroxysuccinimide.

2. Synthesis of hydrophilic porous polymer particles with a uniform particle size

The starting materials, glycidyl methacrylate (3.1 ml), ethylene dimethacrylate (1.9 ml), toluene (7.1 ml), dodecane (0.4 ml), and 2,2'-Azobis(2,4-dimethyl-valeronitrile) 50 mg were passed through an MPG (Micro Porous Glass) pipe with the average pore size of 1.95 μm under pressure and extruded into a 2 wt% polyvinyl alcohol solution to prepare a O/W emulsion. The emulsion was subjected to polymerization at 70°C for 6 hours, and the latex particles with a uniform particle size were in a high yield. The average particle diameter of the latex particles was 12.5μm, the CV (coefficient of variation)value was 12.4%, and the particles were uniform in size. The synthesized latex particles (3.5 g) were dispersed into an aqueous solution (160 ml) containing 1,6-hexyldiamine (1.8 g), and the mixture was stirred at 30°C for 2 hours.

The hydrochloric acid-calcium chloride method (Nakamura et al., Kobunshi Ronbunshu, 38(7) (1981) 485-491.) gave that the latex particles had 3.1 mmol/g of epoxy groups on their surfaces prior to the treatment with 1,6-hexyldiamine. Furthermore, the assay using titration revealed that 0.36 mmol/g of amino groups were introduced onto the surfaces of the hydrophilic porous

polymer particles by the treatment with 1,6-hexyldiamine.

The 1,6-hexyldiamine-treated hydrophilic porous polymer particles (3.5 g) were then added to 10 ml of acetic anhydride, the solution was stirred to acetylate the amino groups, thereby obtaining amidated particles. These amidated particles did not adsorb bovine serum albumin (BSA) in a 20 mM phosphate buffer (pH 7.0) used as a mobile phase. This indicates that the amidated hydrophilic porous polymer particles had a hydrophilicity sufficient to render them suitable as a chromatography carrier (base matrix) protein separation by affinity chromatography.

3. Immobilization of poly(N-isopropylacrylamide) on the support

A mixture containing 4.5 g of the 1,6-hexyldiamine-treated hydrophilic porous polymer particles from the previous partobtained above, 4.5 g of poly(N-isopropylacrylamide) whose carboxyl group at one end of its molecule is esterified with N-hydroxysuccinimide (from part 1 of this example), and 75 ml of acetonitrile was stirred at room temperature for 12 hours. The particles were then washed with acetonitrile, tetrahydrofuran, methanol, and acetone, and dried at room temperature. Elementary analysis revealed that 3.4 wt% of poly(N-isopropylacrylamide) was immobilized on the particles. In addition, to assess the temperature-dependent affinity-controlling capabilities, the packing material (noCB) was prepared by acetylating the remaining amino groups in the support with acetic anhydride.

4. Immobilization of Cibacron Blue F3G-A on the support

A mixture of the poly(N-isopropylacrylamide)-

immobilized support (0.70 g) (from part 3 of this example), either of 1,3-butadiene epoxide (0.09 ml) or ethylene glycol diglycidyl ether (0.21g), and acetonitrile (10 ml) was stirred at 30°C for 1 hour to allow residual amino groups in the support to react with one of the epoxy groups in the diepoxide compound that served as a spacer. The unreacted amino groups of the support were acetylated by adding acetic anhydride (0.11 ml) to the suspension followed by stirring at 30°C for 1 hour. The resulting support on which the spacer and poly(N-isopropylacrylamide) are immobilized was washed with acetonitrile and acetone, and then dried at room temperature.

A mixture of the support on which poly(N-isopropylacrylamide) and spacer are immobilized (0.61 g), aminohexylated Cibacron Blue F3G-A (0.89 g), and water (10 ml) was adjusted to pH 11 with sodium hydroxide and stirred at 25°C for 3 hours to prepare the packing material that is the support on which poly(N-isopropylacrylamide) and Cibacron Blue F3G-A are immobilized. The amount of immobilized Cibacron Blue F3G-A was determined by titration. The packing material (CB-4) prepared using 1,3-butadiene epoxide as a spacer contained 21 μmol/g of Cibacron Blue F3G-A, whereas the packing material (CB-10) prepared using ethylene glycol diglycidyl ether as a spacer 12 μmol/g.

Example 2

1. Filling of the packing materials

Each of the packing materials, noCB, CB-4, and CB-10, was packed in a stainless-steel column of 4.6 mm in inner diameter and 30 mm in length by the wet packing method using water.

2. Assay for the amount of BSA adsorb d by th packing material

The amounts of BSA adsorbed by the respective packing materials were determined at 40°C using a citrate buffer with pH 5 (I=0.01) as a mobile phase and calculated based on the breakthrough curves taking the result obtained at 20°C as a standard. The results are shown in Table 1.

Table 1

Packing material	Amount of adsorbed BSA per gram packing material
noCB	6.7 μg
CB-4	23.4 μg
CB-10	73.8 μg

CB-10 adsorbed more BSA than CB-4, indicating that the length of the spacer between the support and Cibacron Blue F3G-A influences the BSA adsorption. The result also suggested that poly(N-isopropylacrylamide) immobilized on the support does not significantly influence the BSA adsorption.

3. Temperature-dependent affinity control of the packing materials in affinity chromatography

BSA was allowed to be adsorbed at 40°C by Cibacron Blue F3G-A, the ligand of the packing material CB-10. The temperature was then shifted down to 20°C to change the structure of the

stimulus-responsive polymer. It was confirmed that BSA was released from the packing material and eluted in the mobile phase due to the structural change of the polymer. The result is shown in Fig. 1. BSA (111 µg) was loaded onto a column of CB-10 at 40°C using a citrate buffer with pH 5 (I = 0.01) as a mobile phase. The amount of BSA in the eluate was measured by using MICRO BCA™ PROTEIN ASSAY REAGENT KIT (manufactured by Pierce). An excess amount of BSA was eluted in the first 1 to 4 ml aliquot of the eluate. The mobile phase was passed through the column at 40°C until the eluate volume reached 6 ml, confirming that no more BSA is eluted. The flow of the mobile phase was then stopped and the column was cooled at 20°C for 20 minutes. When the mobile phase flow was resumed at 20°C, the BSA adsorbed to the ligand at 40°C was released and eluted from the column (7 to 9 ml of the eluate), which resulted from the structural change of the poly(N-isopropylacrylamide) immobilized on the support. Moreover, the amount of BSA eluted in a temperature-dependent manner was 90% of the total amount of BSA adsorbed by CB-10. These results revealed that a target substance can be removed or separated and purified from a solution using a material comprising a support/base matrix to which a stimulus-responsive polymer and a ligand having affinity for the target substance are covalently attached via separate links. The results also show that the affinity between the target substance and the ligand can be controlled by physical stimulus such as temperature.

Industrial Applicability

The affinity-controlling material according to the

present invention is advantageous in the following points.

1) Since no chemically severe condition is needed in the separation and purification of a target substance, the activity or recovery yield of a physiologically active substance, etc. can be largely elevated compared with the conventional separation/purification methods.

2) Owing to the covalent bonds of the affinitive substance of the target substance and the stimulus-responsive polymer to the support, it is not feared that they might peel off and disturb the separation/purification.

3) When the affinity-controlling material of the present invention is used as an affinity chromatographic packing, the packing can be quickly regenerated compared with the conventional supports.

4) The affinity-controlling material of the present invention makes it possible to separate and purify various types of target substances, which cannot be achieved by the conventional affinity chromatographic packings.

CLAIMS

1. An affinity-controlling material, wherein a stimulus-responsive polymer and an affinitive substance (ligand) having affinity for a target substance are independently attached, preferably covalently, to a support matrix.

2. The affinity-controlling material as claimed in claim 1, wherein the affinity between the affinitive substance and the target substance is possible to change reversibly by subjecting a mixture of the affinity-controlling material and the target substance in solution to a physical stimulus thereby changing the chemical or physical environment around the affinitive substance provided by the polymer.

3. The affinity-controlling material as claimed in claim 1 or 2, wherein the affinity of the affinitive substance of the target substance is reversibly changed by a physical stimulus while keeping at least one of conditions other than temperature constant.

4. The affinity-controlling material as claimed in claim 1, 2 or 3, wherein said physical stimulus is a temperature change.

5. The affinity-controlling material as claimed in any of claims 1, 2, 3, or 4, wherein the affinitive substance of a target substance does not interact with the stimulus-responsive polymer.

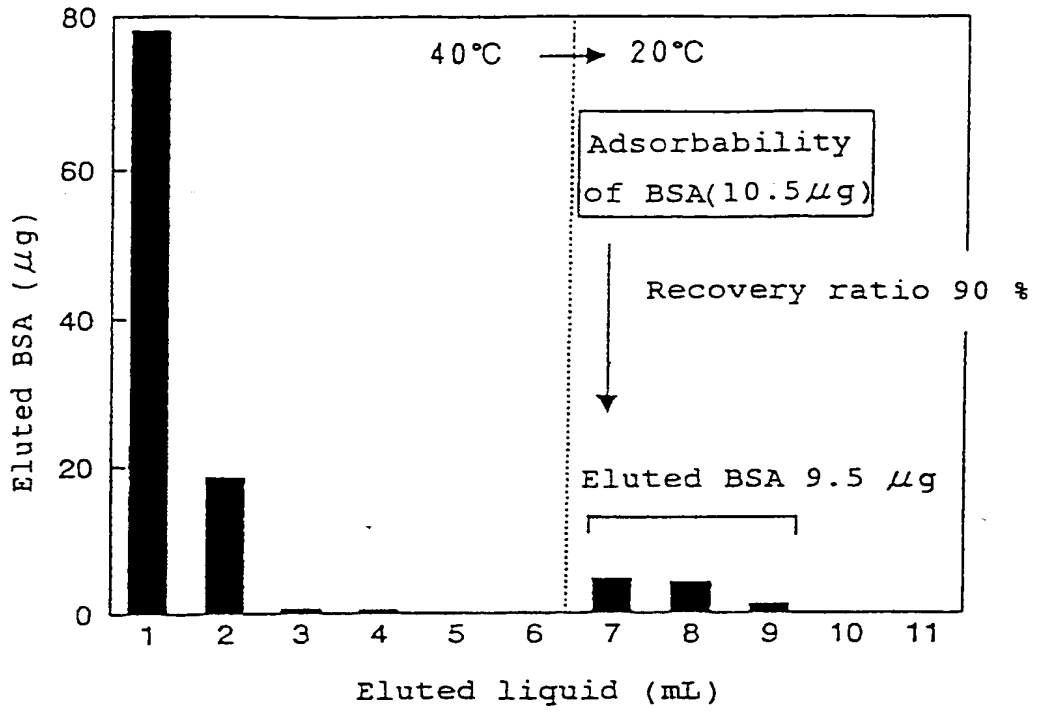
6. The affinity-controlling material as claimed in any of claims 1 to 5, wherein the bonding ability of the target substance is controlled depending on the length of a spacer by which the affinitive substance of the target substance is bonded to the support or the size of the stimulus-responsive polymer.

7. The affinity-controlling material as claimed in any of claims 1 to 6, wherein the support comprises hydrophilic porous polymer particles having a uniform particle size produced by the membrane emulsification method and a chemical treatment with an acidic substance or a basic substance starting with a monomer having epoxy groups in the side chain.

8. The affinity-controlling material as claimed in any of claims 1 to 7 which is to be used as a chromatographic packing.

9. A method for separating and purifying a target substance with the use of the affinity-controlling material as claimed in any of claims 1 to 7.

Fig. 1


80
60
40
20
0
Eluted BSA (μg)
40℃ → 20℃
Adsorbability of BSA(10.5μg)
Recovery ratio 90 %
Eluted BSA 9.5 μg
1
2
3
4
5
6
7
8
9
10
11
Eluted liquid (mL)